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The antihemorrhagic factor of the Mexican ground squirrel, (*Spermophilus mexicanus*)

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Abstract

The Mexican ground squirrel (*Spermophilus mexicanus*) has a natural resistance to western diamondback rattlesnake venom (*Crotalus atrox*). The LD_{50} for the Mexican ground squirrel is 53 mg/kg body weight, which is 13 times higher than that of BALB/c mice. An antihemorrhagic factor from serum of the Mexican ground squirrel was isolated using Sephadex G-200 gel filtration, ion exchange A-50, G-75 gel filtration and HPLC DEAE 5PW ion exchange chromatography. The purified factor neutralized proteolytic and hemorrhagic activity of crude *C. atrox* venom. The results of this research suggest that the antihemorrhagic factor in the serum of the Mexican ground squirrel is not an antibody and neutralizes hemorrhagic activity of *C. atrox* venom.

Resistance to snake venom in mammals has been studied and documented since 1895 (Phisalix and Bertrand, 1895, 1899; Kilmon, 1976; Ovadia and Kochva, 1977; Werner and Vick, 1977; Werner and Faith, 1978; Perez et al., 1978a, 1979; Menchaca and Perez, 1981; De Wit and Westrom, 1985, 1987; Tarng et al., 1986; Perales et al., 1986; Poran et al., 1987; Perales et al., 1992; Pifano et al., 1993; Omori-Satoh et al., 1994, 1998; Rodriguez-Acosta et al., 1995; Neves-Ferreira et al., 1997; Soares et al., 1997). Perez et al. (1978b) showed that 14 of 40 species of warm-blooded animals studied in Texas were able to neutralize the venom of the Western diamondback rattlesnake (*Crotalus atrox*). The Mexican ground squirrel (*Spermophilus mexicanus*) was one of the most resistant.

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The purpose of this study was to purify and characterize antihemorrhagins (metalloproteinase inhibitors) in the serum of the Mexican ground squirrel. The metalloproteinase inhibitors isolated will be useful in studying the mechanism of detoxification of metalloproteinases in venom and other metalloproteinase disease processes.

The antihemorrhagic assay described by Omori-Satoh et al. (1972) was used to measure the ability of Mexican ground squirrel sera to block the hemorrhagic activity of the venom. The antihemorrhagic activity of the Mexican ground squirrel serum and isolated fractions was determined by incubating two minimal hemorrhagic dose (MHD), 5 μg of *C. atrox* venom (the amount of venom resulting in a 10 mm hemorrhagic spot), with an equal volume of the sample to be tested for 1 h at 22°C. After incubation, 0.1 ml of each sample–venom mixture was injected intracutaneously into the depilated back of a New Zealand (*Oryctolagus cuniculus*) rabbit. The rabbit was sacrificed 24 h later, the skin was removed and the cross diameters of the hemorrhagic spots were measured. The crude serum had an antihemorrhagic activity of 64, with a specific activity of 23.

A summary of the techniques that were used in the purification of the Mexican ground squirrel serum is shown in Table 1. At each purification step the antihemorrhagic specific activity increased.

Table 1
Purification of the antihemorrhagic factor from the serum of the Mexican ground squirrel (*Spermophilus mexicanus*)

Step	Concentration mg/ml[a]	Total protein (mg)[b]	Yield (%)[c]	Activity[d]	Specific activity[e]	Purification factor[f]
Crude serum	55	1092	100	64	23	1
G-200[g]	1	202	19	8	160	7
DEAE A-50[h]	0.412	2.5	0.23	4	190	8
G-75[g]	0.082	0.66	0.06	2	490	21
DEAE 5PW HPLC[h]	0.032	0.066	0.006	1	630	27

[a] The protein concentration was measured by Biuret assay at 540 nm. BSA was used as standard protein.
[b] Total protein recovered from each purification step.
[c] The yield was calculated as the total protein recovered divided by total starting protein, multiplied by 100.
[d] The activity (titer) is expressed as the reciprocal of the highest dilution of a sample blocking the MHD (2.5 μg) of *C. atrox* venom.
[e] The specific activity is expressed as the activity divided by the total protein (mg) in each sample in 0.1 ml.
[f] The purification factor is the number of times that the specific activity increased over the crude venom.
[g] The sample was eluted with 0.1 M Tris–HCl buffer, pH 8.0 at a flow rate of 0.5 ml/min.
[h] The sample was eluted with 0.1 M Tris–HCl buffer, pH 8.0 with a 0.5M NaCl gradient at a flow rate of 0.5 ml/min.

Crude Mexican ground squirrel serum and its isolated components were examined for proteolytic activity. Twenty-five μl of crude serum and 25 μl of purified fractions were each applied to gelatin-coated X-ray film. The X-ray film was incubated for 2 h at 37°C in a moist chamber. A transparent spot on the X-ray film indicated gelatinase activity. Crude serum and the purified fractions did not contain proteolytic activity.

Sixty Mexican ground squirrels were trapped in Kleberg County and divided into six groups. Each group was injected with 0.5 ml of *C. atrox* venom at various concentrations. Deaths were recorded at 24 h and the LD_{50} calculated by the method of Reed and Meunch (1938). The LD_{50} for *C. atrox* venom on the *S. mexicanus* was 53 mg/kg. The LD_{50} was determined 15 yr ago by one of the authors, R. Campos, but never published.

Mexican ground squirrel antihemorrhagic factor was incubated for 30 min at various temperatures (25, 35, 45, 55, 65, 75, 85 and 95°C) and then chilled in 0°C water. The antihemorrhagic assay was used to determine the activity of the sample. Thermostability of the antihemorrhagic activity was stable from 0 to 70°C.

The pH stability of the antihemorrhagic activity of crude Mexican ground squirrel serum was also examined at various hydrogen ion concentrations. Crude serum was dialyzed against 0.05 M phosphate buffer of varying pH values (2, 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12). The solutions were kept at 4°C for 24 h and then dialyzed against a 0.05 M phosphate buffer, pH 7.5 for 24 h. Antihemorrhagic activity remained constant from pH 2 to 12.

Pooled crude Mexican ground squirrel serum was tested for the formation of an antigen–antibody complex in a ring precipitation test. The serum was further examined by a gel diffusion test. In this test, goat antivenin was used as a positive control. A precipitate was not formed, which suggests that the antihemorrhagic factor is not an antibody–antigen complex.

The antihemorrhagic factor(s) from the HPLC DEAE 5PW ion exchange separation was subjected to SDS polyacrylamide electrophoresis, using a Pharmacia PhastSystem. The antihemorrhagic factor had a molecular weight of 52 kDa. The isoelectric pH of the antihemorrhagic factor was determined to be 4.9. Only one band was visible in lane D (Fig. 1) when the antihemorrhagic factor was applied to a Pharmacia PhastSystem 8–25 gradient gel.

Many researchers have reported that warm-blooded animals are highly resistant to snake venom, and have antihemorrhagic factors in their sera (Phisalix and Bertrand, 1895, 1899; Kilmon, 1976; Werner and Vick, 1977; Ovadia and Kochva, 1977; Werner and Faith, 1978; Perez et al., 1978a,b, 1979; Menchaca and Perez, 1981; Pichyangkul and Perez, 1981; Garcia and Perez, 1984; De Wit and Westrom, 1985, 1987; Perales et al., 1986; Tarng et al., 1986; Poran et al., 1987; Perales et al., 1992; Pifano et al., 1993; Omori-Satoh et al., 1994, 1998; Rodriguez-Acosta et al., 1995; Neves-Ferreira et al., 1997; Soares et al., 1997). The antihemorrhagic factors are not enzymes since they do not have proteolytic activity. They are not antibodies since their physical characteristics differ from antibodies. Sánchez et al. (1998) showed by a modified western blot that the

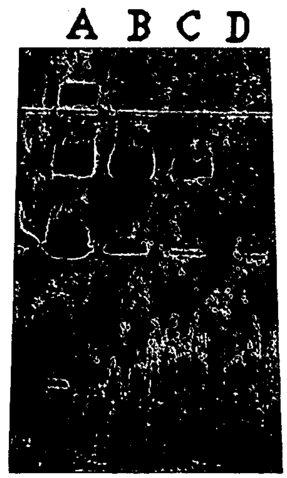

Fig. 1. Polyacrylamide gel electrophoresis separated by a Pharmacia PhastSystem. A µl containing one mg of antihemorrhagic peak from G-200 (lane A), DEAE A-50 (lane B), G-75 (lane C), and Waters HPLC DEAE 5PW (lane D) were applied individually to a Pharmacia Phast gel, gradient 8–25. After separation, the gel was stained with a Pharmacia Phastgel silver kit. The gel shows the successive purification of the antihemorrhagic factor from a heterogenous mix of serum proteins.

antihemorrhagins in opossum serum bind to the hemorrhagins in snake venoms. Ovadia et al. (1977) showed a complex formation between a neurotoxin and its neutralizing protein by Sephadex G-75 chromatography and Catanese and Kress (1992) showed a complex formation between oprin and *C. atrox* α-protease by Mono Q HR 5/5 column chromatography.

De Wit and Westrom (1987) were the first to identify these antihemorrhagic factors as metalloproteinase inhibitors. The isolation of metalloproteinase inhibitors in naturally resistant animals are important in the understanding of the mechanism of neutralization of metalloproteinases in snake venom. These proteinase inhibitors could also have other important biomedical applications.

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